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TECHNICAL MANUSCRIPT 440

GROWTH OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS IN TISSUE CULTURES OF MINCED AEDES AEGYPTI LARVAE

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In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

A method for the germfree cultivation of the mosquitoes Aedes aegypti and Aedes triseriatus was developed, and primary tissue cultures were prepared from minced larvae of both insect species. The Trinidad and the 9t strains of Venezuelan equine encephalitis (VEE) virus and the Louisiana strain of eastern equine encephalitis (EEE) virus were grown in larval tissue cultures of A. aegypti. The Trinidad strain of VEE virus was also grown in A. triseriatus larval tissue cultures. The growth of VEE virus in A. aegypti larval tissue culture was influenced by the length of time, the temperature, and the virus concentration used for the adsorption process, and the temperature, pH, and agitation of cultures during the growth process. In these cultures, the Trinidad strain grew somewhat better than the 9t strain; its latent period was shorter by about 8 hours, its growth rate was faster, and it reached higher maximal virus titers. However, EEE virus was superior to the Trinidad strain in each of these growth characteristics. The Trinidad strain, the 9t strain, and the Louisiana strain attained maximal titers of 7.4, 6.1, and 7.6 $\log_{10}$ pfu/ml, respectively. The presence of a viral inactivating material was detected in larval tissue cultures of both species of mosquitoes. Ten serial passages of the Trinidad strain or five serial passages of the 9t strain in A. aegypti larval tissue cultures caused no detectable changes in either the mouse virulence or plaque size distribution of both virus strains.

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TABLES

I. INTRODUCTION

The growth characteristics of many arboviruses have been studied in a variety of living vertebrates and vertebrate cell systems, but comparatively little information has been published on the arthropod phase of development of these viruses. Mussgay[1] reviewed much of the information then available on the growth and transmission of arboviruses in living arthropods and on the growth characteristics of these viruses in vertebrate cell cultures, but he did not include studies in arthropod tissue culture. Because arthropod tissue cultures have been difficult to establish, very few publications have reported the use of these cultures for the growth of viruses. In 1938, Trager[2] showed that western equine encephalitis virus could multiply to a significant extent in hanging drop tissue cultures of imaginal discs from larvae of the mosquito Aedes aegypti. Much later, in 1963, Peleg and Trager[3] reported the multiplication of West Nile virus in similar preparations. Haines[4] observed in 1958 that eastern equine encephalitis virus could be maintained for several days but it did not appear to multiply in surviving midgut tissue cultures of A. aegypti larvae. Rehacek,[5] using primary cell monolayers of trypsinized tissues from ixodid ticks, reported the multiplication of a number o rboviruses in tick cultures, and Rehacek and Pesek[6] found these cells to be more susceptible than chick fibroblasts to infection by tick-borne encephalitis virus.

More recently, Suitor[7] reported the limited growth of Japanese encephalitis virus in cultures of a continuous insect cell line derived by Grace[8] from tissues of the silk moth Antheraea eucalypti, and Converse and Nagle[9] obtained growth of yellow fever virus in this cell line as well as in another continuous cell line, also derived by Grace,[10] from tissues of the mosquito A. aegypti.

The difficulties inherent in the preparation of arthropod tissue cultures have been a major factor in the slow development of this type of virus research. However, recent studies of the physiology of arthropods and the development of methods for the axenic rearing of many arthropods have made the preparation of maintenance cultures of arthropod tissues somewhat less difficult. The present report describes the use of germfree larvae of A. aegypti and Aedes triseriatus for the preparation of Maitland-type tissue cultures and the use of these cultures to determine some of the growth characteristics of two strains of Venezuelan equine encephalitis (VEE) virus and one strain of eastern equine encephalitis (EEE) virus.

II. MATERIALS AND METHODS

A. VIRUS STRAINS

The Trinidad strain[11] and the 9t strain[12] of VEE virus and the Louisiana strain[13] of EEE virus were used in these studies. The Trinidad strain, which was isolated from an infected mule in Trinidad in 1943, has been passed 13 times in embryonated eggs and once in minced chick embryo tissue culture before being used in the present studies. It was passed one additional time in chick fibroblast (CF) monolayer cultures, and the supernatant fluids from these cultures were pooled and used as stock Trinidad virus. The 9t strain was derived from an L cell culture that had become chronically infected with the Trinidad strain of VEE virus. The ninth L cell passage of this virus was passed once in CF monolayer cultures, and the supernatant fluids from these cultures were pooled and used as the stock 9t virus. The Louisiana strain of EEE virus originated from a human infection in Louisiana in 1948 and had been passed eight times in mice and five times in embryonated eggs before being used in these studies. It too was passed once in CF monolayer cultures, and the supernatant fluids were pooled and used as stock EEE virus. All strains of virus were stored in screw-capped vials at -70 C in 2-ml amounts.

B. MOSQUITO STRAINS

The Rockefeller strain of A. aegypti and the Alabama strain of A. triseriatus mosquitoes were employed. Eggs were surface sterilized using the Roccal-Clorox procedure of Lea, Dimond, and DeLong.[14] After hatching in diluted beef heart infusion broth (BHIB), the mosquito larvae were incubated overnight at room temperature to check for sterility and were placed in 250-ml flasks (about 400 to 500 larvae per flask) containing 100 ml of a modified Akov's medium.[15] Akov's medium was modified by the substitution of 1.0% autoclaved brewers' yeast for casein and by the addition of 0.1% liver extract (Nutritional Biochemicals, Inc.) and 1.0% sucrose. The flasks were incubated in an insectary at 26 C and 50% relative humidity.

C. TISSUE CULTURE

Fourth instar mosquito larvae were filtered from the growth medium using sterile, fine-mesh marquisette and washed several times with sterile distilled water. They were transferred to a sterile beaker, drained of fluid, and minced with scissors. The tissue obtained in this manner was washed twice with an insect balanced salt solution (IBSS) of Bradford and Ramsey[16] and suspended in a mosquito tissue culture medium of Clements.* This medium was a modification of a Lepidoptera cell culture medium reported by Grace.[8]

* Personal communication.

D. VIRUS ADSORPTION

Minced tissue, obtained from about 5,000 larvae, was suspended in 5 ml of Clements' medium containing a suitable amount of virus. The mixture was shaken in a Dubnoff shaker at 120 cycles per minute at 30 C for 1 hour. After washing several times with IBSS to remove unadsorbed virus, the tissue was distributed among five 50-ml flasks. Each flask then received 20 ml of Clements' medium containing 5% bovine serum and was incubated at a suitable temperature with continuous shaking. Samples of supernatant fluid were removed at appropriate intervals, placed in BHIB containing 1% bovine serum, and kept frozen at -70 C until assayed. Samples were assayed in CF monolayer cultures using a plaque procedure described by Colon et al.[17]

III. RESULTS

A. GROWTH OF GERMFREE MOSQUITOES

Because Akov's growth medium did not provide sufficient nutrients to support a very dense population of mosquito larvae, and because casein used as a protein source interfered with the separation of larvae from the growth mixture, some modifications of the medium were advisable. Akov suggested brewers' yeast as a substitute for casein because it gave somewhat better growth rates. It could also be autoclaved, and it formed a finely dispersed suspension from which the larvae could be easily separated. The growth media reported by Lea et al.[14] and by Trager[2] served as bases for the use of sucrose and liver extract, respectively. In the modified growth medium finally adopted, both species of mosquito larvae grew well with high population densities. After 6 days of incubation, most larvae had developed to the fourth instar, and a few had already begun to pupate. By the 8th day a majority of the insects had developed to the pupal stage, and by the 9th day adults began emerging. Synchronization of the germfree cultures was not as good as that obtained using optimal conditions in a normal environment, but it was adequate to provide substantial numbers of larvae for virus studies.

B. ADSORPTION OF VIRUS

Studies of the adsorption of VEE virus to mosquito larval tissue were complicated by several factors: (i) large amounts of chitin and other debris in the tissue culture could not be separated from living cellular material, (ii) a viral inactivating factor was present, the concentration of which could not be measured, and (iii) the amount of viable tissue could not be estimated with any reasonable degree of accuracy. For these reasons, the usual methods for measuring adsorption could not be used.

However, over a limited range of virus concentrations, the viral titer of a culture at 72 hours was related to the amount of virus present in the adsorption mixture (Fig. 1). This observation was used as an indirect, approximate measure of the amount of adsorption taking place in these cultures. Adsorption in this case must be defined as the amount of virus that adsorbed to, penetrated, and replicated in viable cells. The effects on the adsorption process of time, temperature, and agitation of cultures could then be determined in at least a semiquantitative manner.

Adsorption of VEE virus to larval tissue at 30 C was rapid (Table 1). By 10 minutes, a significant amount of virus had adsorbed to the tissue, and by 30 minutes, adsorption was essentially complete. No further adsorption or elution of the virus could be detected at 60 minutes.

The temperature range for efficient adsorption was between 4 C and 30 C. Table 2 shows that over this range of temperature, the amount of virus adsorbed to mosquito larval tissue in 1 hour appeared to be constant, as indicated by the constancy of virus titers after 72 hours of growth. Adsorption at 37 C, however, was greatly reduced and produced very little growth of virus, the titer increase being less than 10-fold as compared with more than a 1,000-fold increase for samples adsorbed at lower temperatures.

As shown in Table 3, agitation of the cultures during the virus adsorption process appeared to have no significant effect on the amount of virus adsorbed, but it markedly affected the growth of the adsorbed virus. Cultures adsorbed with virus while stationary or with agitation and grown under stationary conditions developed less than 10-fold increases in virus titers, but similar cultures, when agitated during the growth phase, showed greater than 100-fold increases in virus titers at 72 hours.

C. GROWTH OF VIRUS

The growth of VEE virus was noticeably affected by the concentration of virus used in the adsorption mixture, as mentioned previously. To study this effect, replicate cultures, each containing tissue from about 2,000 larvae, were adsorbed with various amounts of VEE virus and incubated at 30 C. The culture supernatant fluids were assayed for virus concentration at intervals, and the results were plotted against time as shown in Figure 1. As the virus concentration in the adsorption mixture was decreased below about 5.6 $\log_{10}$ pfu/ml, the appearance of new virus was progressively delayed, until, with an inoculum concentration of about 2.4 $\log_{10}$ pfu/ml, no new virus was produced even though the cultures were incubated for 6 days. Cultures that did produce virus attained a level of infectivity of about 8.0 $\log_{10}$ pfu/ml on or before the 5th day. Varying the virus concentration in the adsorption mixture affected the time of appearance of new virus but did not greatly influence the peak level of infectivity attained.

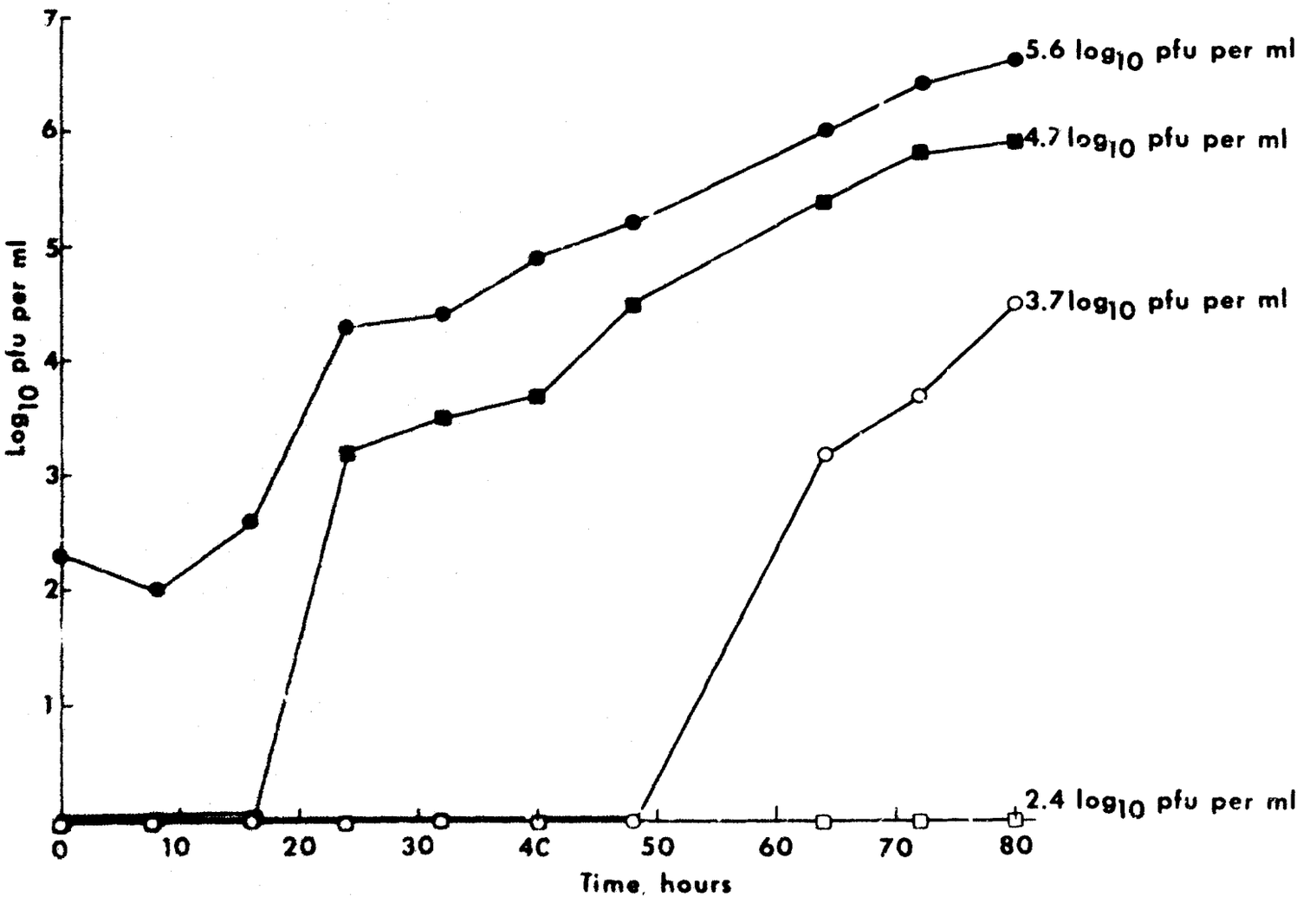

Figure 1. Effect of Virus Concentration during Adsorption on Growth of the Trinidad Strain of Venezuelan Equine Encephalitis Virus in Aedes aegypti Larval Tissue Culture.

TABLE 1. RATE OF ADSORPTION[a] OF THE TRINIDAD STRAIN OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS[b] TO AEDES AEGYPTI LARVAL TISSUES AT 30 C

Time of Incubation, hours	Infectivity, $\log_{10}$ pfu/ml, at Indicated Adsorption Time, min						
	1	10	20	30	40	50	60
0	2.5	2.5	2.7	2.8	2.5	2.5	2.5
72	2.0	3.3	4.0	4.3	4.2	4.4	4.3

a. See text for method and explanation of adsorption.
b. 6.3 $\log_{10}$ pfu/ml in adsorption mixture.

TABLE 2. EFFECT OF TEMPERATURE ON ADSORPTION[a] OF THE TRINIDAD STRAIN OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS[b] TO AEDES AEGYPTI LARVAL TISSUES

Time of Incubation, hours	Infectivity, $\log_{10}$ pfu/ml, at Indicated Adsorption Temperature, C				
	4	15	25	30	37
0	2.5	2.9	2.8	2.9	3.0
72	6.2	6.4	6.3	6.3	5.9

a. Time of adsorption, 1 hour. See text for method.
b. 6.5 $\log_{10}$ pfu/ml in adsorption mixture.

TABLE 3. EFFECT OF AGITATION ON GROWTH OF THE TRINIDAD STRAIN OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS IN MOSQUITO LARVAL TISSUE CULTURES

Incubation Time, hr	Growth	Infectivity, $\log_{10}$ pfu/ml Adsorption	
		Stationary	Shaking
0	-	5.8	5.5
72	Stationary	5.6	6.0
72	Shaking	7.5	7.7

The effect of temperature on the growth of VEE virus was determined by adsorbing larval tissues with either the Trinidad or the 9t strain of virus for 1 hour at 30 C and incubating the cultures at 25, 30, or 37 C. Virus concentrations were measured in the culture fluids at various time intervals and plotted as shown in Figure 2 for the Trinidad strain and Figure 3 for the 9t strain. After an initial decrease in titer lasting about 18 hours for the Trinidad strain and about 24 hours for the 9t strain, the virus concentration increased rapidly for the next 48 to 96 hours before becoming constant. The rate of viral replication was related to the temperature of incubation, increasing as the temperature increased. The effect of temperature on growth rate was most pronounced with the Trinidad strain. The growth rates at higher temperatures with both strains are actually greater than are indicated in the figures when the rate of viral inactivation in cell-free media at each temperature is taken into consideration. Throughout the temperature range studied, replicate cultures of the Trinidad strain usually attained approximately equal maximal titers regardless of the temperature used for incubation, although these peak titers were maintained for only a very short time at the highest temperature used. At 37 C, the 9t strain grew rather poorly, reaching its maximal titer in less than 3 days and decreasing sharply thereafter. The rate of decrease in titer paralleled the virus inactivation curve at 37 C, suggesting that virus multiplication ceased after about 72 hours at this temperature. This observation is consistent with the report by Brown[18] that the 9t strain had a lower growth temperature threshold than the Trinidad strain.

Within limits, the amount of mosquito tissue per ml of culture fluid seemed to have relatively little effect on the virus growth; over the range of 20 to 200 larvae per ml of medium, the growth rate and the maximal titer of the Trinidad strain of VEE virus were essentially constant. However, cultures containing more than about 100 larvae per ml of medium showed a noticeable loss in titer during the first 18 to 24 hours. This initial drop in titer became more marked as the concentration of tissue was increased, suggesting that a component inactivating for the virus might be present in mosquito tissue cultures.

Because published reports[19,20] indicate that arthropod tissue cultures survive best in media buffered between pH 6 and pH 7, the effect of this variable on the yield of VEE virus was studied by growing the virus in cultures buffered at various pH values. Larval tissues were infected with the Trinidad strain of VEE virus and placed in media buffered at values from pH 6.0 to 8.0 in 0.5 pH units. Samples of supernatant fluid were taken at intervals throughout the growth period and assayed for viral infectivity. In these experiments, viral growth was most rapid at pH 6.0 and 6.5, reaching a peak titer in 3 days. At pH 7.0, the growth rate was somewhat slower than that at pH 6.5 and gradually decreased as the pH of the medium was increased. The time required to reach maximal titer increased with pH, but the final titers reached in any given experiment were similar regardless of the pH of the suspending medium.

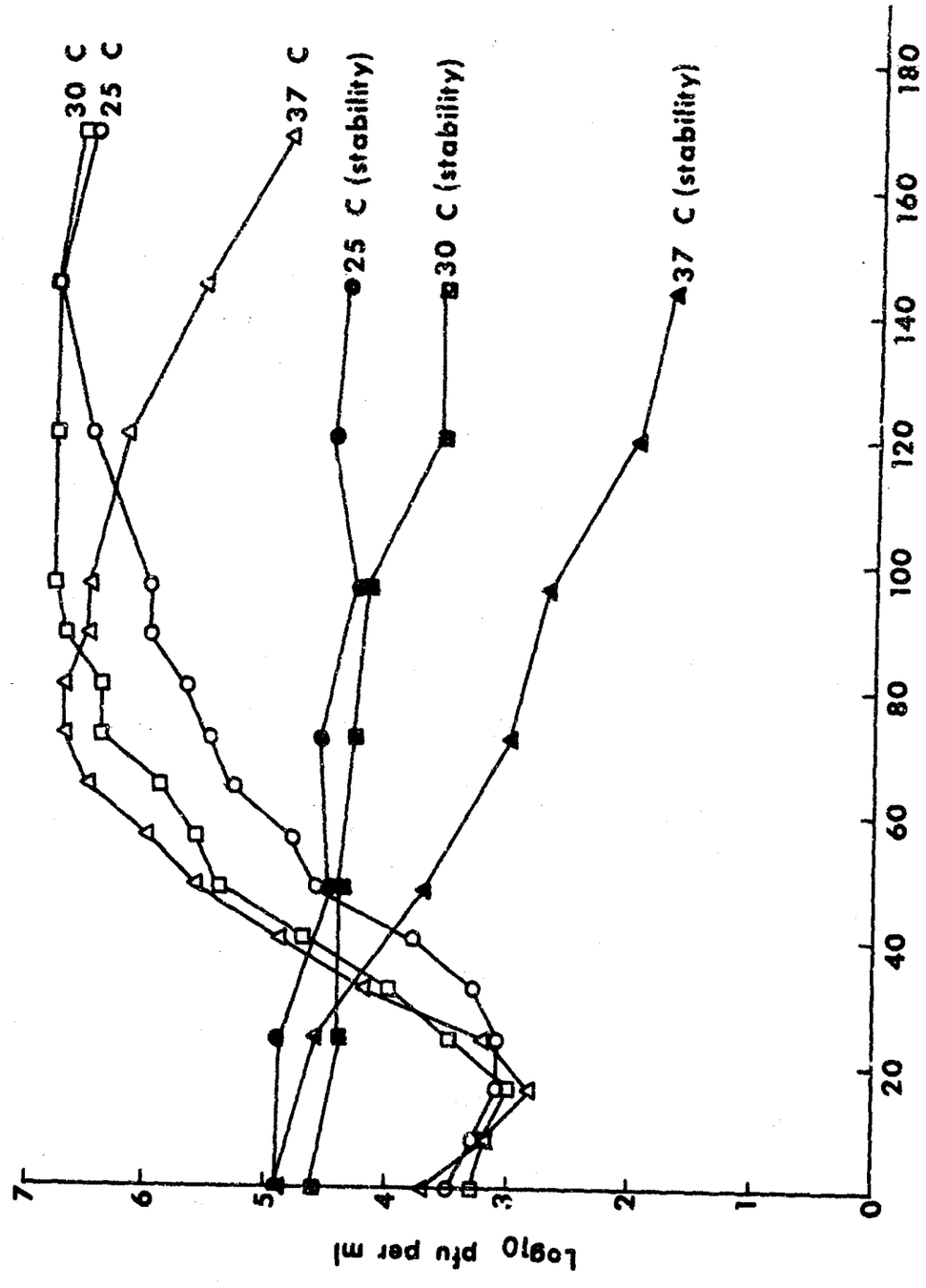

Figure 2. Effect of Temperature on Growth and Stability of the Trinidad Strain of Venezuelan Equine Encephalitis Virus in Mosquito Larval Tissue Culture.

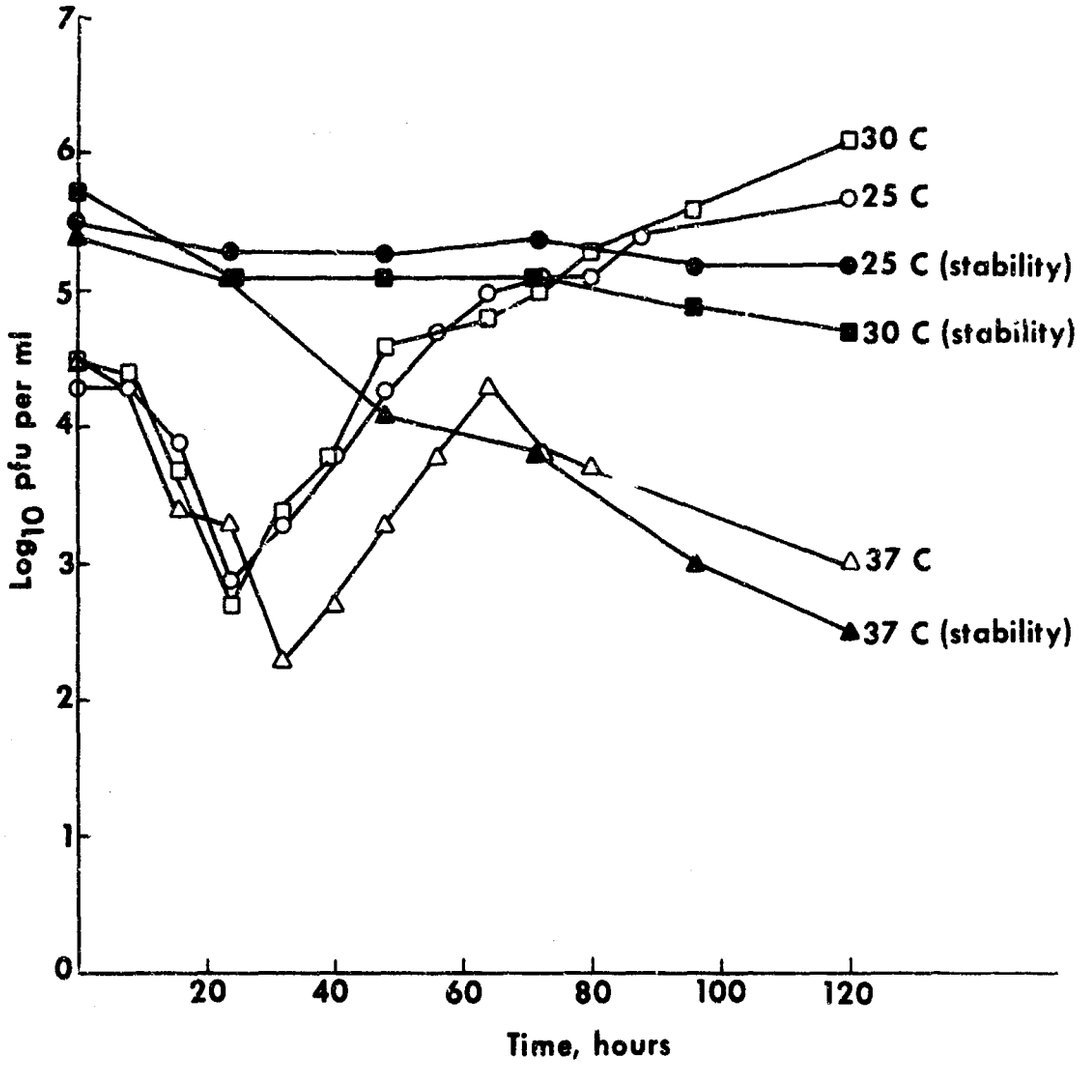

Figure 3. Effect of Temperature on Growth and Stability of the 9t Strain of Venezuelan Equine Encephalitis Virus in Mosquito Larval Tissue Culture.

In 1961, Hardy and Brown[31] reported that, in L cell cultures of VEE virus, the infectivity titer in the supernatant fluid was equal to or higher than the titer found in the cells. Similar observations have been made with other arboviruses, by Brown* with EEE virus, and by Rubin, Baluda, and Hotchin[32] with western equine encephalitis virus. To determine whether this would also be true for VEE viral growth in mosquito larval tissues, a series of cultures was prepared, infected with VEE virus, and incubated at 30 C. At intervals, supernatant fluid and tissue from these cultures were separated by centrifugation, and the virus concentration in each fraction was determined. The results of these experiments are shown in Figure 4. Unlike virus growth in L cell cultures, the supernatant fluid of mosquito larval cultures contained less virus than the cells. Throughout most of the growth curve, the cell-associated viral infectivity was about 0.5 log higher than that of the supernatant fluid.

D. TISSUE SURVIVAL

Growth curves of VEE virus in mosquito tissue culture usually showed a steady decline in titer after 5 or 6 days of incubation. To determine whether a decrease in cell viability independent of viral infection was the cause of this decline, the following experiment was performed. A series of replicate cultures of larval tissues was incubated at 30 C with shaking. At 2-day intervals, a culture flask was removed and the tissue was separated from the medium and infected with the Trinidad strain of VEE virus by adsorption for 1 hour at 30 C. The tissue was washed, placed in fresh medium, and reincubated at 30 C. Samples of supernatant fluid were taken at 0 and 3 days after infection and assayed for virus. The results of these experiments are shown in Table 4. From these data, it appears that larval tissue remained viable in cultures for at least 8 days at 30 C because it could be infected and could produce significant amounts of virus after this time. Because most experiments reported here were terminated in 6 or 7 days, the culture medium appeared capable of maintaining the tissue for at least this length of time, and "normal" physiological cell death was probably not the limiting factor in the decline in production of virus. Because of the difficulties inherent in working with insect tissue cultures, procedures such as dye exclusion or cloning could not be used for measuring cell viability. It is felt, however, that the ability to infect tissues with a virus is as good a criterion of cell viability as other methods.

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* Brown, Arthur, personal communication.

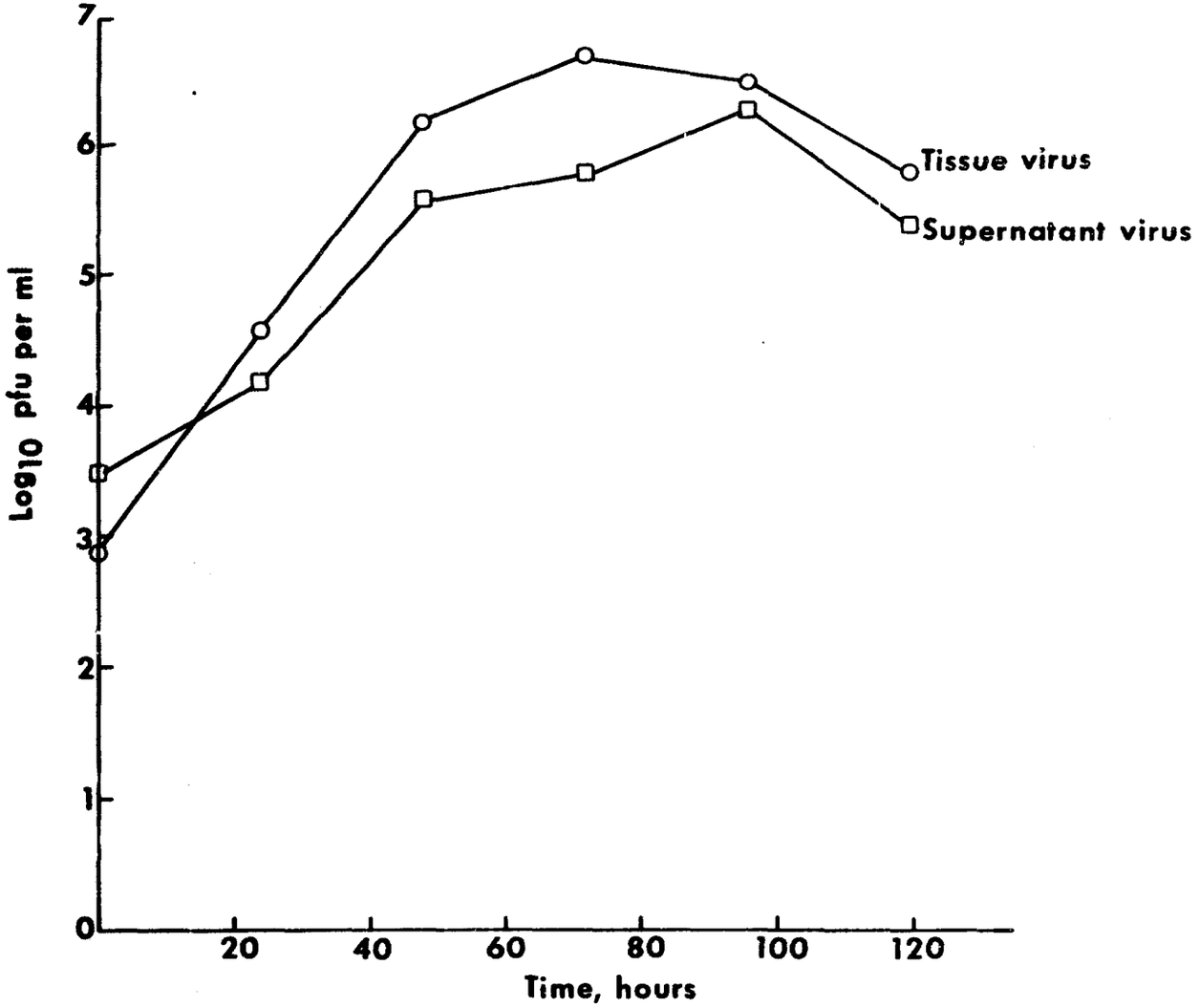

Figure 4. Concentrations of Venezuelan Equine Encephalitis Virus in Tissues and Supernatant Fluids of Tissue Culture of Aedes aegypti Larvae at 30 C.

TABLE 4. SURVIVAL OF MINCED TISSUE CULTURES OF LARVAE OF THE MOSQUITO AEDES AEGYPTI[a]/

Days of Incubation[b]/	Virus Titer after Adsorption with VEE Virus, $\log_{10}$ pfu/ml	
	0 Day	3 Day
0	3.5	6.3
4	3.5	6.4
6	3.4	6.2
8	3.5	6.2

a. Tissue culture viability was determined by its capacity to permit multiplication of Venezuelan equine encephalitis virus.
b. Time of incubation at 30 C before adsorption with VEE virus.

To determine whether VEE virus killed mosquito cells during its growth in tissue culture, the following experiments were performed. Tissue cultures of A. aegypti larvae were infected with VEE virus as described and allowed to incubate for 7 days at 30 C with continuous shaking. The supernatant fluids were removed, and the tissue was washed several times, resuspended in fresh medium, and reincubated. The same process was repeated at 11 days and 19 days. Samples of culture supernatant fluids were taken at intervals and virus titers were determined. The results are shown in Table 5. These data indicate that virus infection did not seriously affect tissue viability for at least 11 days, if viability is measured by the capacity to produce a new virus. By 19 days, however, the tissues could no longer produce significant amounts of additional virus. Because it was not technically feasible to use other methods for measuring viability in these cultures, the tissues were considered to be no longer viable.

TABLE 5. SURVIVAL OF *AEDES AEGYPTI* LARVAL TISSUE CULTURES[a] AT 30 C AFTER INFECTION WITH THE TRINIDAD STRAIN OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS

Day of Testing	Infectivity of Supernatant Fluid, $\log_{10}$ pfu/ml		
	Initial Titer	Titer after Media Change	
		0 hr	96 hr
0	5.3	3.5	6.0
7	6.5	3.5	5.8
11	5.8	4.4	6.5
19	6.5	4.5	4.7

a. Tissue culture viability was determined by its capacity to permit virus multiplication after media change.

E. GROWTH OF EEE VIRUS

Because VEE virus grew readily in tissue cultures of mosquito larvae, it was of interest to compare its growth with that of another virus, EEE, under the same conditions. Tissue cultures of *A. aegypti* larvae were prepared and infected with EEE virus in experiments conducted in a manner similar to those described previously for the effect of temperature on VEE virus. The results of these experiments are shown in Figure 5. Growth curves obtained with EEE virus were, in general, similar to those obtained with the Trinidad strain of VEE virus, but several differences were apparent. The latent period of EEE virus, the time period before new virus can be detected, appeared to be shortened, in some cases, by as much as 8 hours. EEE virus also showed slightly more rapid growth rates at all temperatures used, and peak titers were significantly higher than those of VEE virus. The stability curves for EEE virus showed a generally greater rate of inactivation than those for VEE virus, suggesting that differences in growth rates and peak titers between the two viruses may be greater than these figures indicate.

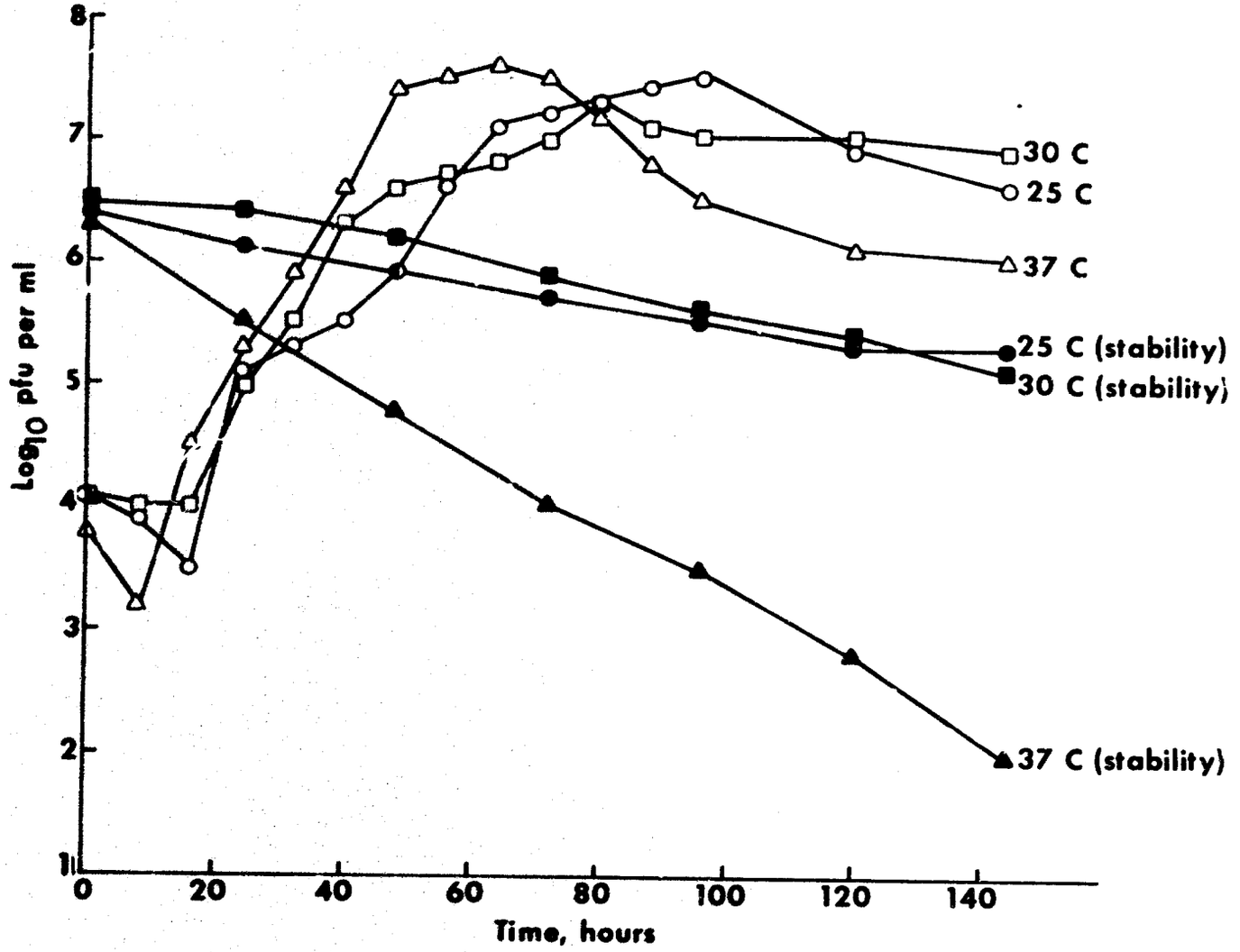

Figure 5. Growth and Stability Curves of Eastern Equine Encephalitis Virus in Aedes aegypti Larval Tissue Culture.

F. LATENT VIRUSES

Attempts were made to determine whether latent viruses could be detected in the larvae of A. aegypti. Germfree larvae were homogenized and inoculated intracerebrally into suckling mice, into the yolk sac and amniotic sac of developing chick embryos, and into CF monolayer cultures. In no case was there any evidence of the reactivation of a latent virus infection. Minced cultures of larvae were incubated at 30 C for 3, 7, 10, and 14 days, and samples from the whole culture were ground in a mortar and inoculated as previously described into mice, eggs, and CF cell cultures. These experiments were also negative for the presence of a reactivated latent virus. Finally, tissue cultures of larvae were incubated for 1 week at 30 C, and the whole culture was homogenized and mixed with fresh larval tissue. This process was repeated at 2 and 3 weeks, and samples of whole culture homogenates from each passage were again inoculated into mice, eggs, and CF cell cultures. These experiments were also negative. From these data, it appeared that the mosquito stock was free of obvious latent viruses.

G. GROWTH IN A. TRISERIATUS TISSUE CULTURES

Larval tissue cultures of A. aegypti mosquitoes supported the growth of VEE virus readily, so it was of interest to determine whether similar cultures prepared from a related mosquito, A. triseriatus, would also support growth of this virus. A. triseriatus larvae were reared under germfree conditions, and tissue cultures prepared from them were infected with the Trinidad strain of VEE virus in a manner similar to that described for A. aegypti cultures. In these cultures, VEE virus exhibited growth patterns similar to those observed in A. aegypti larval cultures. Growth rates were similar, and peak titers were at about the same level. Because viral growth characteristics were similar in tissue cultures of both species of mosquitoes, A. triseriatus, being a larger insect, may be of some use in the preparation of larger scale tissue cultures.

H. PASSAGE STUDIES

Both strains of VEE virus were serially passed in mosquito larval tissue cultures; the Trinidad strain was passed ten times and the 9t strain five times. No major changes in growth patterns occurred with either strain on repeated passage, although slight increases in growth rates and maximal titers were observed as the virus became adapted to the new host system. The mouse virulence and the plaque size distribution of the two strains of virus were determined before and after

passage in larval tissue culture. The mouse virulence, as measured by the ratio of the intraperitoneal LD_{50} to intracerebral LD_{50}, and the plaque size distribution were not altered after ten passages of the Trinidad strain or five passages of the 9t strain in mosquito larval tissue culture. This is in contrast to the reports of Mussgay,[1] Hearn and Soper,[23] Brown,* and others, who reported changes toward decreased virulence and an increase in the percentage of small plaque types with repeated passage in vertebrate cell culture.

IV. DISCUSSION

The growth characteristics of VEE virus in mosquito larval tissue culture differed in several respects from growth characteristics reported for this virus in vertebrate cell cultures. In insect tissue culture, there was a marked loss of viral titer during the initial 18 to 24 hours of incubation, a loss much greater than that occurring in vertebrate cell cultures.[11] Virus growth was also much slower, as indicated by the time required for the culture to reach its peak titer. Finally, maximal titers were about 10-fold lower in insect than in vertebrate cell cultures.

The initial loss of virus titer in insect tissue cultures, the degree of which seemed to be related to the temperature and the amount of tissue in the culture, suggested that a factor, inhibitory or inactivating to the virus, might be present. A further indication of such a factor appeared when it was found that the virus concentration in the tissue portions of mosquito cultures was higher than that in the supernatant fluids. Because Hardy and Brown[21] have already demonstrated that VEE virus is released from L cells as rapidly as it is formed, this could suggest that virus was being inactivated after release from the mosquito cells. The presence of an inactivating factor for VEE virus in mosquito tissue cultures would influence the growth of virus in several ways. A continuous destruction of virus could increase its apparent latent period, lower its measured growth rate, and reduce the peak titer that would be reached in these cultures. It could also account for the increase in the minimal amount of virus needed to infect a mosquito tissue culture.

It is interesting that ten serial passages in A. aegypti larval tissue culture had relatively little effect on those properties of the Trinidad strain of VEE virus that were studied. After passage in insect tissue culture, virulence for mice was essentially identical to that of the parent strain, and the plaque size distribution also remained unaltered. Mussgay[1] cites a number of references that suggest that growth of arboviruses in a variety of cell culture systems tends to reduce the virulence of the agent and frequently increases the percentage of small plaque types. In view of these studies, mosquito tissue cultures, especially the larval cultures, may provide a growth system that will maintain a given set of viral characteristics over at least a limited number of passages.

* Brown, Arthur, personal communication.

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13. ABSTRACT

A method for the germfree cultivation of the mosquitoes Aedes aegypti and Aedes triseriatus was developed, and primary tissue cultures were prepared from minced larvae of both insect species. The Trinidad and the 9t strains of Venezuelan equine encephalitis (VEE) virus and the Louisiana strain of eastern equine encephalitis (EEE) virus were grown in larval tissue cultures of A. aegypti. The Trinidad strain of VEE virus was also grown in A. triseriatus larval tissue cultures. The growth of VEE virus in A. aegypti larval tissue culture was influenced by the length of time, the temperature, and the virus concentration used for the adsorption process, and the temperature, pH, and agitation of cultures during the growth process. In these cultures, the Trinidad strain grew somewhat better than the 9t strain; its latent period was shorter by about 8 hours, its growth rate was faster, and it reached higher maximal virus titers. However, EEE virus was superior to the Trinidad strain in each of these growth characteristics. The Trinidad strain, the 9t strain, and the Louisiana strain attained maximal titers of 7.4, 6.1, and 7.6 $\log_{10}$ pfu/ml, respectively. The presence of a viral inactivating material was detected in larval tissue cultures of both species of mosquitoes. Ten serial passages of the Trinidad strain or five serial passages of the 9t strain in A. aegypti larval tissue cultures caused no detectable changes in either the mouse virulence or plaque size distribution of both virus strains.

14. Key Words		
*Tissue culture	*Venezuelan equine encephalitis Eastern equine encephalitis	*Aedes aegypti *Aedes triseriatus Arboviruses

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